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Article

In *Arabidopsis thaliana* Heterosis Level Varies among Individuals in an F_1 Hybrid Population

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Abstract: Heterosis or hybrid vigour is a phenomenon in which hybrid progeny exhibit superior yield and biomass to parental lines and has been used to breed F_1 hybrid cultivars in many crops. A similar level of heterosis in all F_1 individuals is expected as they are genetically identical. However, we found variation of rosette size in individual F_1 plants from a cross between C24 and Columbia-0 accessions of *Arabidopsis thaliana*. Big sized F_1 plants had 26.1% larger leaf area in the 1st and 2nd leaves than medium sized F_1 plants at 14 days after sowing in spite of the identical genetic background. We identified differentially expressed genes between big and medium sized F_1 plants by microarray; genes involved in the category of stress response were overrepresented. We made transgenic plants overexpressing 21 genes, which were differentially expressed between the two size classes, some lines had increased plant size at 14 or 21 days after sowing but not at all time points. Change of expression levels in stress responsive genes among individual F_1 plants, implying epigenetic changes, could generate the variation in plant size of individual F_1 plants in *A. thaliana*.

Keywords: Heterosis; Hybrid vigor; Transcriptome

1. Introduction

Heterosis or hybrid vigour is the superior performance of F_1 (heterozygous) plants relative to their inbred (homozygous) parental lines. In the process of plant breeding, the phenomenon of heterosis has been exploited in various crops and vegetables because of its effect on yield or stress tolerance [1]. Hybrid breeding has been remarkably successful starting with maize [2,3], but the molecular mechanism of heterosis remains unknown. Several genetic models have been hypothesized for the explanation of heterosis [1,4-6]. The dominance model explains that heterosis is due to the complementation of deleterious recessive alleles by favorable dominant alleles at multiple loci. The overdominance model argues that the heterozygous state leads to superior performance of hybrids to either homozygous condition. The epistasis model is that interaction of favorable alleles at different loci results in heterosis. Epigenetic modifications are also considered to contribute to heterosis; interactions between parental epigenetic states in the two sets of chromosome in hybrids play a role in heterosis [5,7,8].

The concept that the superior performance of hybrid is caused by establishment of more favorable gene expression levels relative to the parental lines has been considered [9]. The transcriptome profile has been compared between hybrids and parental lines in a number of

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heterotic hybrids of maize, rice, and *Arabidopsis thaliana* [10-18]. Though the majority of genes show an additive gene expression pattern, differentially expressed genes between hybrids and the mid-parent value (MPV), termed non-additively expressed genes, are detected [1]. In some studies, non-additively expressed genes involved in specific functional categories have been suggested to play a role in the heterosis phenotype, while there are reports showing that the majority of non-additively expressed genes are not associated with any specific categories [10-19].

In addition to crops and vegetables, *A. thaliana* also shows substantial heterosis in vegetative biomass in particular parental combinations [20-25], and several approaches such as quantitative trait locus (QTL) analysis, transcriptome, metabolome, small RNAome, and epigenome analysis have been used to identify genes and mechanisms that may be important for heterosis [1,8]. In the hybrid between C24 and Columbia-0 (Col) accessions of *A. thaliana*, heterosis is obvious at early developmental stages in increased cotyledon area at a few days after sowing. Larger cotyledon size generates an increase in photosynthetic capacity, suggesting that this increased photosynthetic capacity in hybrids may cause the maintenance and/or magnification of heterosis at later developmental stages [14]. A similar phenotype at early developmental stages has been observed in other parental combinations of *A. thaliana* [26].

In this study, we found variation in plant size among individual hybrids between C24 and Col accessions. To identify the genes regulating the altered plant size in individuals with the same genetic background, we compared the transcriptome profile between big and medium sized F_1 plants using microarrays. A number of genes showed a higher expression level in the big sized F_1 plants than in the medium sized F_1 plants; we examined their effect on plant size by overexpression, focusing on genes categorized into "transcription factor". A number of transgenic plants were larger at 14 or 21 days after sowing, suggesting that these genes play a part in the control of plant size.

2. Results

2.1. There is variation in shoot size in the F_1 between C24 and Col

The F_1 between C24 and Col had a heterosis phenotype in shoots [14,15]. Among 80 F_1 plants, shoot size evaluated by rosette diameter at 14 days after sowing (DAS) varies, and the biggest rosette diameter is 2.7 times larger than smallest (Figure 1A). The size of dry seed evaluated by seed area also showed variation within the seventy-two F_1 seeds, and the seed area of the biggest seed is 1.5 times larger than the smallest (Figure 1B).

To examine whether the larger shoot size in F_1 plants is due to larger seed size, we examined the relationship between shoot and seed sizes. There is no difference in rosette diameter at 14 DAS of nine F_1 plants derived from small, medium, and big seed fractions (Figure 1C), indicating that the difference of seed size is independent from shoot size in the F_1 population.

Using twenty-two plants of each of the big (14.1% larger) and medium size fractions in rosette diameter at 10 DAS from 103 F_1 plants (Figure 2A), the rosette diameter, leaf area, and the size of first layer of palisade mesophyll cell in the 1st and 2nd leaves were examined at 14 DAS. At 14 DAS, F_1 plants that have been big at 10 DAS retained the larger rosette diameter (15.2%) relative to the medium sized F_1 plants (Figure 2B), and the big sized F_1 plants had 26.1% larger leaf area in the 1st and 2nd leaves relative to the medium sized F_1 plants (Figure 2C). The big sized F_1 plants had a 21.6% reduction in the number of the first layer of palisade mesophyll cells per unit area relative to the medium sized F_1 plants at 14 DAS (Figure 2D), indicating that the larger leaf area of the big sized F_1 plant is due to the increased cell size.

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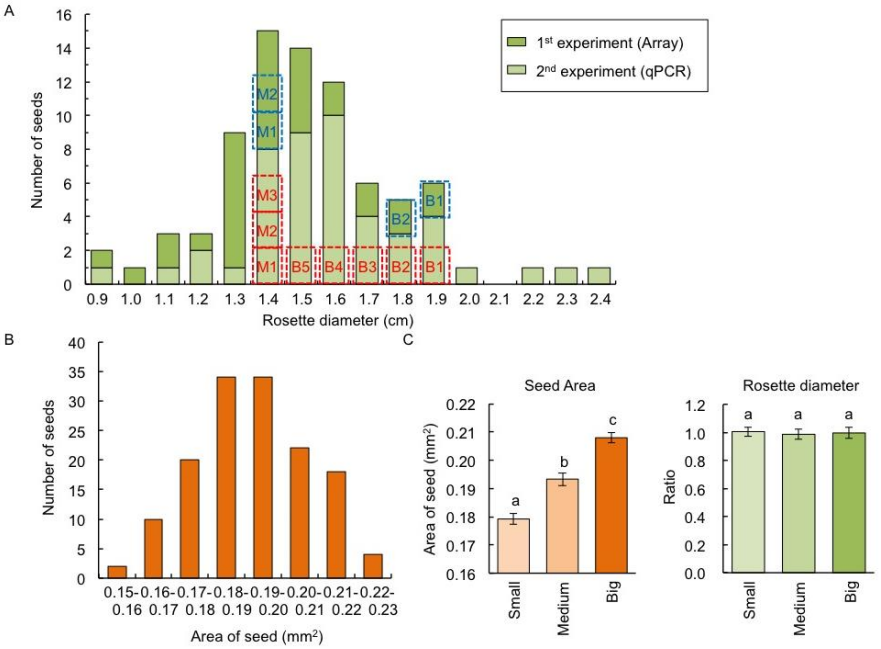

Figure 1. Variation of rosette diameter and seed size among individual F_1 plants between C24 and Col. (A) Distribution of rosette diameter among individual F_1 plants (n=80) at 14 DAS. Dark and light green bars represent plant materials used for microarray analysis and quantitative RT-PCR (qPCR), respectively. Blue and red dotted lines represent the plant size used for microarray and qPCR, respectively. (B) Distribution of seed area in F_1 (n=72). (C) The seed area (left panel) and rosette diameter at 14 DAS (right panel) derived from small, medium, and big seed fractions. The ratio of the rosette diameter compared with plants derived from big seed fractions are shown. Data presented are the average and standard error (s.e.) (n=9). Letters above the bars indicate significant differences at $p < 0.05$ (Tukey-Kramer test).

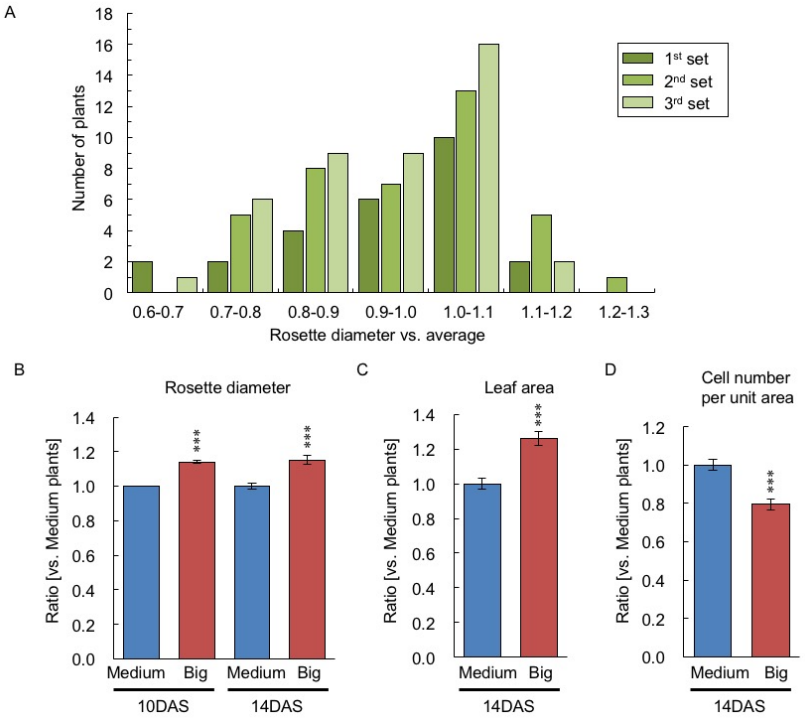

Figure 2. Comparison of the rosette diameter and true leaf area and its cell number per unit area between medium and big sized F_1 plants. (A) Distribution of ratio of rosette diameter compared with average of rosette diameter in F_1 plants (n=103) at 10 DAS. Three replicates were performed

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represented as 1st, 2nd, and 3rd sets. (B) Ratio of rosette diameter compared with medium sized F_1 plants in big sized F_1 plants at 10 and 14 DAS. (C) Ratio of leaf area of 1st and 2nd leaves compared with medium sized F_1 plants in big sized F_1 plants at 14 DAS. (D) Ratio of cell number per unit area in the first layer of palisade mesophyll cell compared with medium sized F_1 plants in big sized F_1 plants at 14 DAS. Data presented are the average and standard error (s.e.) (each, n=22). ***$p < 0.001$ (Student's *t*-test)

2.2. The transcriptome divergence between big and medium sized F_1 plants

We examined the whole genome transcriptome of big and medium sized F_1 plants at 14 DAS, using the Affymetrix, Arabidopsis ATH1 Genome Array using total RNAs from shoots of the biggest (B1, two 1.9cm rosette diameter plants), the second biggest (B2, two 1.8 cm rosette diameter plants), and two medium size fractions (M1 and M2, two 1.4 cm rosette diameter plants) of plants (Figure 1A). We did not use small sized F_1 plants, as there is a risk that decrease in plant size is due to disease or failure to thrive. The 441 probe-sets showed 1.5-fold difference with 5% false discovery rate (FDR) in expression between B1&B2 and M1&M2 (Table S1). Among differentially expressed genes, 361 (81.9%) probe-sets were expressed at a higher level in B1&B2 than M1&M2 (B1&B2 > M1&M2 expression), and 80 probe-sets showed B1&B2 < M1&M2 expression (Table S1).

We compared the lists of non-additively expressed genes in aerial tissues between three F_1 hybrids (C24/Col, C24/Landsberg *erecta* (L*er*), Col/L*er*) and their mid parent values (MPV) at 15 DAS [17], with our lists of differentially expressed genes (Table 1). About 30% of genes showing B1&B2 > M1&M2 expression overlapped with upregulated genes in C24/Col or C24/L*er* hybrids (Table 1), and 61 genes (16.9%) overlapped with upregulated genes in both C24/Col and C24/L*er* hybrids (Table S2). While 5.8% of genes overlapped with upregulated genes in Col/L*er* hybrids (Table 1). Of genes showing B1&B2 < M1&M2 expression, more genes overlapped with downregulated genes in C24/Col hybrids (31.3%) followed by C24/L*er* (22.5%) and Col/L*er* (13.8%) hybrids (Table 1)

Table 1. Number of genes overlapping with previous transcriptome data

		B1&B2 > M1&M2 (361)		B1&B2 < M1&M2 (80)	
		Number	Percentage	Number	Percentage
C24/Col DEG	F_1 > MPV (863)	102	28.3%	8	10.0%
	F_1 < MPV (1,234)	53	14.7%	25	31.3%
C24/L*er* DEG	F_1 > MPV (669)	114	31.6%	10	12.5%
	F_1 < MPV (1,050)	47	13.0%	18	22.5%
Col/L*er* DEG	F_1 > MPV (464)	21	5.8%	7	8.8%
	F_1 < MPV (907)	63	17.5%	11	13.8%
C24xCol vs. *ddm1*C24x*ddm1*Col	WT > *ddm1* (73)	3	0.8%	0	0.0%
	WT < *ddm1* (1,128)	25	6.9%	0	0.0%
ColxC24 vs. *ddm1*Colx*ddm1*C24	WT > *ddm1* (69)	0	0.0%	4	5.0%
	WT < *ddm1* (1,208)	36	10.0%	0	0.0%

We also compared our data with lists of differentially expressed genes between C24xCol and *ddm1*C24x*ddm1*Col hybrids or between ColxC24 and *ddm1*Colx*ddm1*C24 hybrids (Table 1), which showed different plant size with the same genetic background [27]. Twenty-five genes showing B1&B2 > M1&M2 expression overlapped with downregulated genes in C24xCol hybrids compared with *ddm1*C24x*ddm1*Col hybrids (Table 1), including *ethylene responsive element binding factor 5* (*ERF5*), *ERF6*, *ERF105*, *WRKY40*, *DRE BINDING PROTEIN 1B* (*DREB1B*), *salt-inducible zinc finger 1* (*SZF1*), and *SZF2* (Figure S1).

2.3. Confirmation of differential gene expression by quantitative RT-PCR

We confirmed the expression patterns of 24 differentially expressed genes (13 genes, B1&B2 > M1&M2 expression; 11 genes, B1&B2 < M1&M2 expression) using quantitative RT-PCR analysis (qPCR) using another set of big (B1, two 1.9 cm rosette diameter plants; B2, two 1.8 cm rosette

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diameter plants) and medium sized (M1 and M2, two 1.4 cm rosette diameter plants) F_1 plants (Figure 1A). The relative expression levels between B1&B2 and M1&M2 seen in microarray data were highly correlated with those in qPCR ($r = 0.94$, $p < 1.00E-10$) (Figure 3, Table S3).

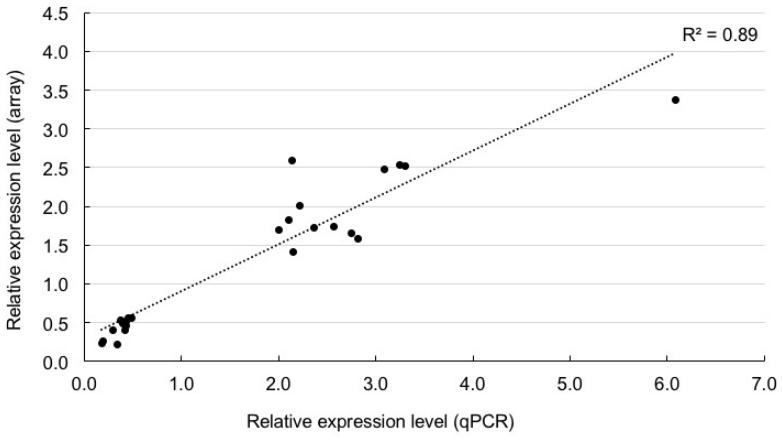

Figure 3. Verification of microarray data by qPCR. Relationship of relative expression levels between qPCR and microarray in 24 differentially expressed genes between big and medium sized F_1 plants.

The expression levels of the 24 genes were analyzed by qPCR in differently sized F_1 plants; B3, two 1.7 cm rosette diameter plants; B4, two 1.6 cm rosette diameter plants; B5, two 1.5 cm rosette diameter plants, and M3, two 1.4 cm rosette diameter plants together with B1, B2, M1, and M2 samples (Figure 1A). We categorized gene expression pattern into eight classes, (I) Expression levels of B1 and B2 were higher than mean expression level of M1, M2, and M3 (B1&B2 > M1-3 expression) (4 genes); (II) B1-3 > M1-3 expression (7 genes); (III) B1-4 > M1-3 expression (1 gene); (IV) B1-5 > M1-3 expression (1 gene); (V) B1&B2 < M1-3 expression (1 gene); (VI) B1-3 < M1-3 expression (4 genes); (VII) B1-4 < M1-3 expression (2 genes); (VIII) B1-5 < M1-3 expression (4 genes) (Figure 4, Table S4). Of 13 genes with B1&B2 > M1&M2 expression (class I to IV), 11 genes (84.6%) were categorized into group (I) and (II), suggesting that the border line of differential gene expression level between big and medium sized F_1 plants was between B3 and B4 (Figure 4A, Table S4). By contrast, there was no clear demarcation in genes with B1&B2 < M1&M2 expression (Figure 4B, Table S4).

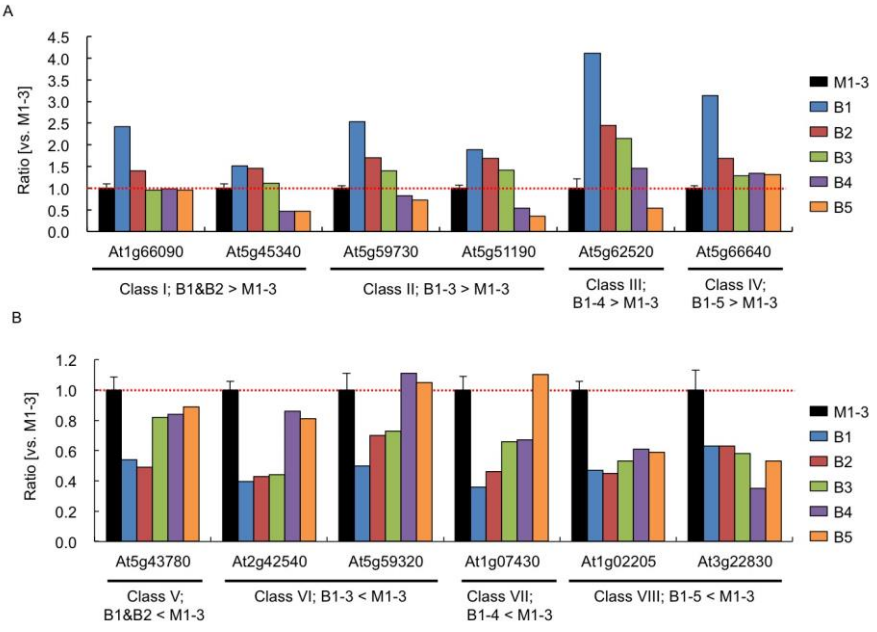

Figure 4. Pattern of gene expression levels in four fractions of big sized F_1 plants and four factions of medium sized F_1 plants. B1 to B5 shows top five fractions from biggest sized F_1 plants. Expression pattern was categorized into eight classes described in text.

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2.4. Comparison of gene expression between big and medium sized F_2 plants

Rosette diameter at 14 DAS varied in F_2 plants derived from the hybrid between C24 and Col (Figure 5A). We examined whether the same genes showed differential gene expression between big (BF_2, two 1.5 cm and one 1.4 cm rosette diameter plants) and medium (MF_2, three 1.1 cm rosette diameter plants) fractions of F_2 plants as in F_1 (Figure 5A). The 14 and 11 genes showing B1&B2 > M1&M2 and B1&B2 < M1&M2 expression, respectively, were used. In the first F_2 big fraction, 6/14 and 4/11 genes showed $BF_2 > MF_2$ and $BF_2 < MF_2$ expression, respectively (Figure 5B, Table S5). In the second F_2 fraction, 9/14 and 4/11 genes showed $BF_2 > MF_2$ and $BF_2 < MF_2$ expression, respectively, (Figure 5B, Table S5). 5/14 and 2/11 genes showed $BF_2 > MF_2$ and $BF_2 < MF_2$ expression, respectively, in both sets (Figure 5B, Table S5), indicating that the same genes (more than 35%) as in the F_1 tended to have a higher expression level in the big sized F_2 plants than in the medium sized F_2 plants.

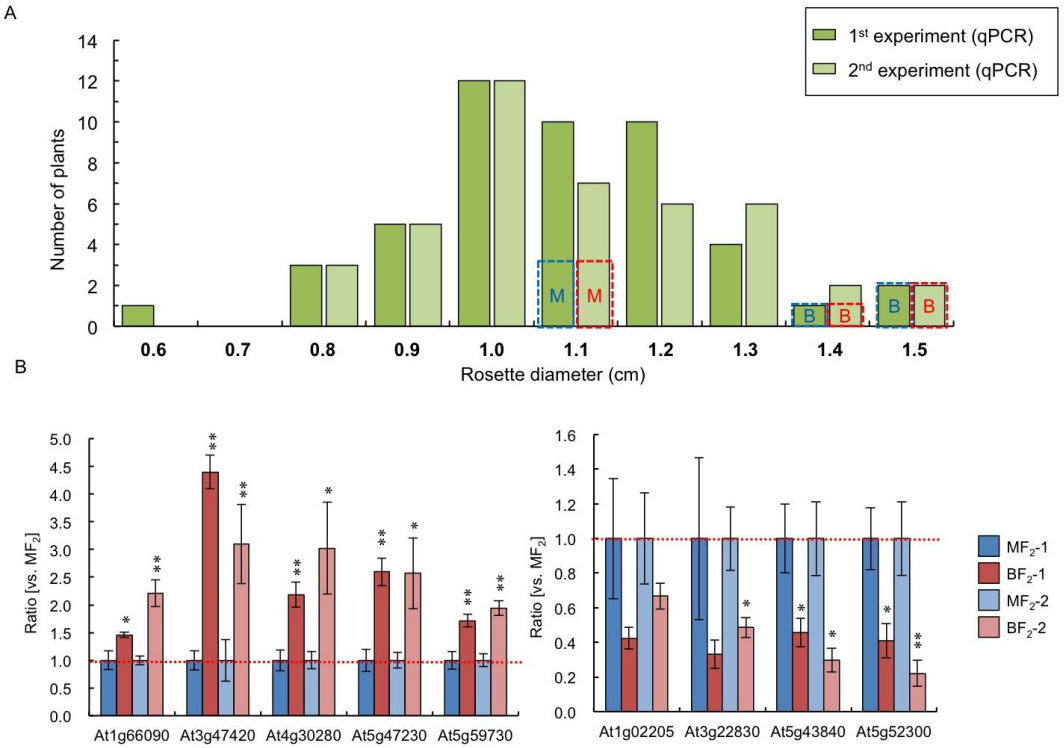

Figure 5. Comparison of the gene expression level between big and medium sized F_2 plants. (A) Distribution of rosette diameter at 14 DAS of F_2 plants. Blue and red dotted lines represent the plant size used for qPCR. (B) qPCR using genes that showed B1&B2 > M1&M2 (left panel) and B1&B2 < M1&M2 expression (right panel). The ratio of the expression levels compared with medium sized F_2 plants in big sized F_2 plants is shown. Data presented are the average and standard error (s.e.) from three biological and experimental replicates. *p<0.05, **p < 0.01 (Student's t-test).

2.5. Classification of the differentially expressed genes between big and medium sized F_1 plants

We categorized the differentially expressed genes between B1&B2 and M1&M2 into GO cellular component, GO molecular function, and GO biological process (Table S6, S7). The categories of 'Response to abscisic acid stimulus', 'Defense response', and 'Response to jasmonic acid stimulus' in GO biological process were overrepresented in genes showing B1&B2 > M1&M2 and B1&B2 < M1&M2 expression (Figure 6, 7, Table S6, S7). In the genes showing B1&B2 > M1&M2 expression, genes categorized into 'Response to chitin', 'Response to ethylene stimulus', 'Response to wounding', and 'Response to salicylic acid stimulus' in GO biological process were overrepresented (Figure 6, 7, Table S6). In the genes showing B1&B2 < M1&M2 expression, genes categorized into 'Leaf senescence', 'Response to salt stress', 'Response to abiotic stimulus', and 'Response to cold' in GO biological process were overrepresented (Figure 6, 7, Table S7). In the 61 genes, which showed

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B1&B2 > M1&M2 expression and upregulated genes in both C24xCol and C24x*Ler* hybrids (Table S2), similar categories to B1&B2 > M1&M2 expressed genes were overrepresented (Figure 6, Table S8).

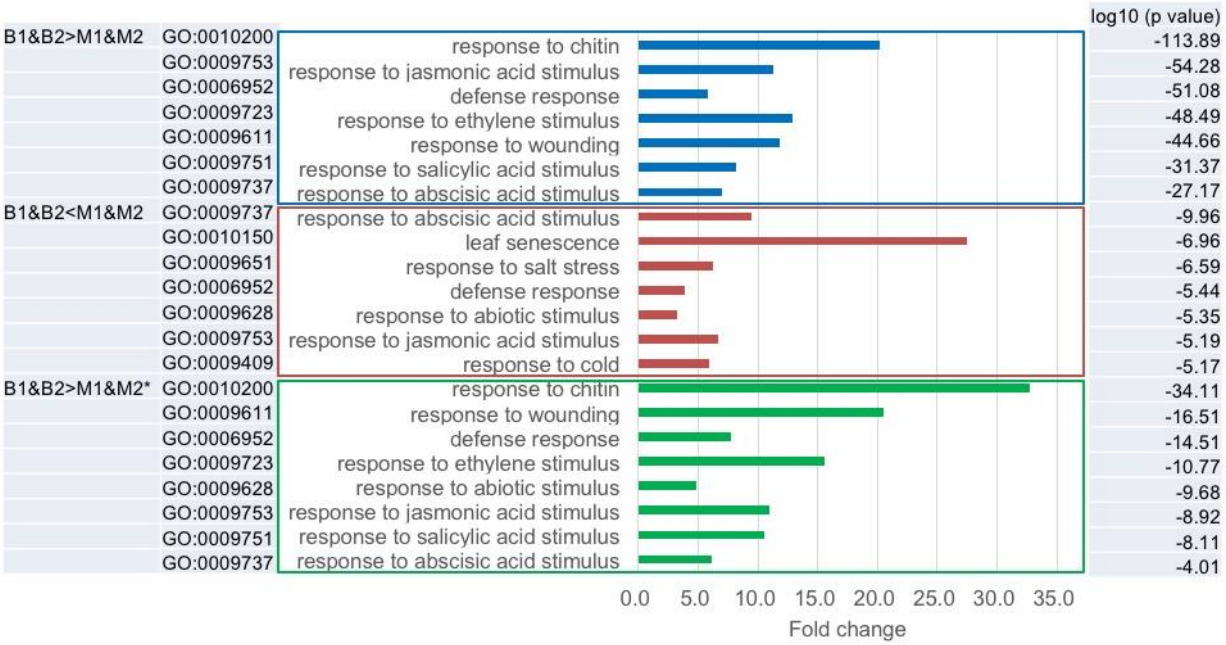

Figure 6. GO classification in B1&B2 > M1&M2 and B1&B2 < M1&M2 expressed genes. * represents overrepresented GO categories in B1&B2 > M1&M2 expressed genes and differentially expressed genes in C24/Col and C24/L*er* hybrids compared with MPV.

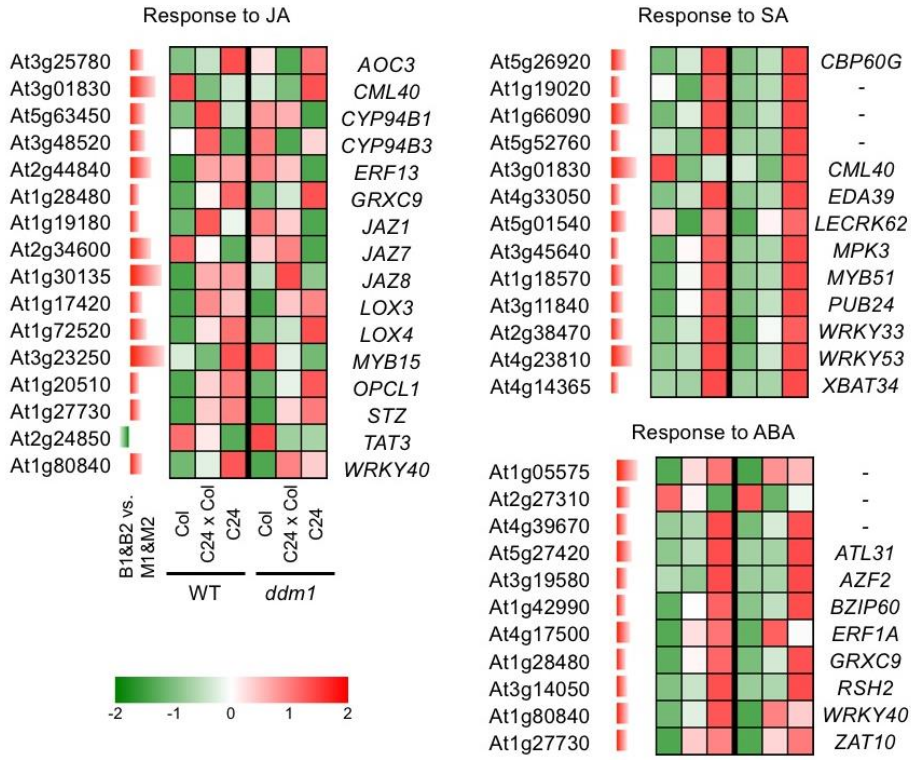

Figure 7. Expression pattern of genes showing B1&B2 > M1&M2 expression. GO terms related to plant hormone response are shown. Expression levels in Col, C24, C24xCol, *ddm1*Col, *ddm1*C24, *ddm1*C24x*ddm1*Col are derived from [27]. Different red/ green colors indicate the fold change (up/down) from the MPV. ABA, abscisic acid; JA, jasmonic acid; SA, salicylic acid.

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2.6. Overexpression resulted in plant size difference

We focused on the B1&B2 > M1&M2 expressed genes, especially transcription factors as they control many biological processes by regulating gene expression. cDNAs from the first methionine to the stop codon of 21 genes were placed under the control of a 35S promoter, and binary vectors were transformed into the wild type Col accession of *A. thaliana* (Table 2). We obtained more than three independent T_1 transgenic plants for each gene. Bulked T_2 seeds were sown on MS medium, and we compared the rosette diameter at 14 and 21 DAS of T_2 plants with and without transgenes. Twelve of 21 lines showed no difference between transgenic plants and non-transgenic controls at either 14 or 21 DAS (Figure 8, Table 2). In two lines, #18 and #20, the rosette diameter of transgenic plants was larger than non-transgenic plants at 14 DAS (Figure 8, Table 2). Three lines, #1, #6, and #14, had larger rosette diameter than non-transgenic plants at 14 DAS, but the rosette diameter was smaller at 21 DAS (Figure 8, Table 2). In three lines, #4, #5, and #13, the rosette diameter in transgenic plants was larger than non-transgenic plants only at 21 DAS (Figure 8, Table 2). A transgenic line, #9, showed a smaller rosette diameter than non-transgenic plants at both 14 and 21 DAS, and plants had pleiotropic seedling size (Figure S2). Although there was no rosette size difference in #19 compared with non-transgenic controls, all plants with transgenes showed narrow and light green leaves (Figure S2). We confirmed the overexpression in some genes compared with control plants (Figure S3).

Table 2. List of genes for producing overexpressed transgenic plants

Number	Gene model	Name	Discription	14 DAS[#]	21 DAS[#]
#1	At1g05575		Unknown protein	1.10**	0.88**
#2	At1g19210	*ERF17*	Encodes a member of the DREB subfamily A-5 of ERF/AP2 transcription factor family	1.00	0.95
#3	At1g22810	*ERF19*	Encodes a member of the DREB subfamily A-5 of ERF/AP2 transcription factor family	1.07	1.06
#4	At1g33760	*ERF22*	Encodes a member of the DREB subfamily A-5 of ERF/AP2 transcription factor family	1.08	1.04*
#5	At2g26020	*PDF1.2b*	Predicted to encode a PR (pathogenesis-related) protein	1.04	1.14*
#6	At2g35290	*SAUR79*	SMALL AUXIN UPREGULATED RNA 79	1.19**	0.88**
#7	At2g44840	*ERF13*	ETHYLENE-RESPONSIVE ELEMENT BINDING FACTOR 13	0.95	0.93
#8	At4g17490	*ERF6*	ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR 6	1.06	1.02
#9	At4g30290	*XTH19*	XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 19	0.92**	0.65**
#10	At4g38840	*SAUR14*	SMALL AUXIN UPREGULATED RNA 14	1.01	0.99
#11	At5g07100	*WRKY26*	Encode WRKY DNA-binding protein 26	0.99	0.90
#12	At5g09570		Cox-19-like CHCH family protein	1.02	1.08
#13	At5g27420	*CNI1*	CARBON/NITROGEN INSENSITIVE 1	0.95	1.06**
#14	At5g42380	*CML37*	Calmodulin like 37	1.16**	0.85**
#15	At5g47230	*ERF5*	ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR 5	1.03	1.05
#16	At5g51190	*ERF105*	Encodes a member of the ERF subfamily B-3 of ERF/AP2 transcription factor family	1.02	0.93
#17	At3g02040	*GDPD1*	GLYCEROPHOSPHODIESTER PHOSPHODIESTERASE 1	0.94	1.00
#18	At3g23250	*MYB15*	MYB DOMAIN PROTEIN 15	1.22**	0.91
#19	At4g34410	*RRTF1/ERF109*	REDOX RESPONSIVE TRANSCRIPTION FACTOR 1	1.03	0.92
#20	At2g20750	*EXPB1*	EXPANSIN B1	1.24**	1.06
#21	At4g28250	*EXPB3*	EXPANSIN B3	0.98	0.98

#, ratio of rosette diameter in transgenic plants compared with non-transgenic plants. *, $p < 0.05$; **, $p < 0.01$ (Student's *t*-test)

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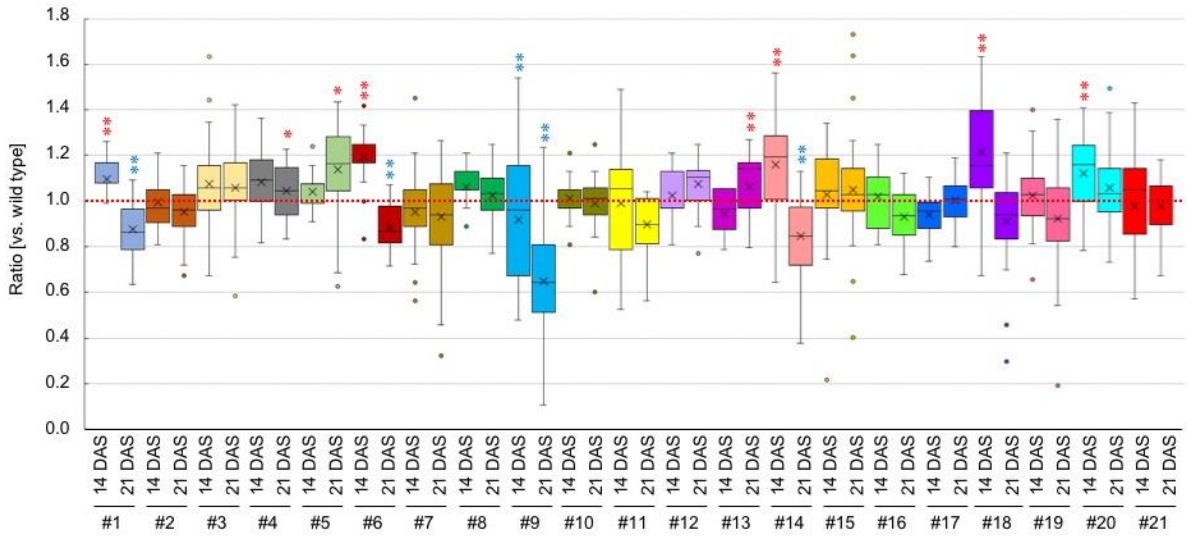

Figure 8. Rosette diameter of overexpressed plants. y-axis shows the ratio of rosette diameter in transgenic plants compared with non-transgenic plants. *p<0.05, **p < 0.01 (Student's t-test).

3. Discussion

One of the advantages of F_1 hybrid cultivars in crops and vegetables is their uniform phenotype, which makes management of cultivation easier [28]. This uniformity of F_1 hybrid cultivars is considered to be due to the high rate of homozygosity of the parental lines. As *A. thaliana* has a high rate of inbreeding [29], the genetic background in the C24 x Col hybrid should be identical in individual plants and C24 x Col hybrids show a similar level of heterosis in each. However, our results showed variation of plant size among individual F_1 plants; the biggest rosette diameter is more than two times larger than the smallest. This increased leaf area in big sized F_1 plants is due to increased cell size. These results suggest the possibility that heterosis level and/or plant size are affected by epigenetic changes because of their identical genetic background. One possibility is that there is epigenetic variation within individual F_1 plants. Non-additive DNA methylation occurs in the F_1 by the allelic interaction of different DNA methylation states in parental lines [30,31], and the DNA methylation state might not be uniform in individual F_1 plants. The epigenetic inbred (epiRIL) lines, which have a difference of DNA methylation level with the same genetic background, show higher divergence of flowering time and plant height compared to wild type Col [32]. This phenomenon could explain plant size variation in individual F_1 plants. The other possibility is that epigenetic variation was generated by environmental effects. One candidate is differences of light intensity as in this hybrid, there is increased heterosis under increased light intensity [21]. In our experiments, plants were grown under well-controlled conditions and differences of light intensity between plants were small (150–180 μmol photons·m−2·s−1), but this small difference might result in plant size of F_1 plants varying.

We identified differentially expressed genes between big and medium sized F_1 plants, and approximately 30% of genes showing B1&B2 > M1&M2 expression overlapped with genes upregulated in heterotic F_1 plants (C24/Col and C24/L*er*) compared with MPV. These genes were categorized into response to wounding, defense response, and response to plant hormones such as ethylene (ET), abscisic acid (ABA), salicylic acid (SA), and jasmonic acid (JA). Transcriptome analysis comparing heterotic F_1 and MPV suggested several possibilities to explain the heterosis. (1) Decreased in expression levels of defense responsive genes may play an important role in heterosis by reducing energy cost for defense and releasing resource allocation to plant growth [17,24,33]. (2) A reduction in SA concentration with lower expression levels of SA responsive genes is associated with increased biomass [17]. (3) Negative effects of ET on heterosis have been suggested [34]. (4) Delayed senescence could be involved in heterosis at later developmental stages [35]. In our transcriptome data, similar categories were overrepresented in differentially expressed genes

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between big and medium sized F_1 plants. However, sometimes the direction of change of expression levels does not match between big vs. medium sized F_1 plants and the heterotic F_1 vs. MPV, i.e., in big sized F_1 plants, more defense responsive genes were upregulated, and higher expression levels in genes involved in response to ET or SA stimulus was found. These results suggest that these genes are involved in determining the plant size, and any further increase in plant size of heterotic F_1 plants may result from a different expression pattern to non-additive expression.

Loss of DDM1 function showed a decreased in heterosis level compared with wild type F_1 [27, 36]. The 25 genes showing B1&B2 > M1&M2 expression overlapped with downregulated genes in wild type F_1 compared with *ddm1* F_1, i.e., an opposite expression pattern. The relationship between increase / decrease in expression level and plant size does not necessarily match in the big size F_1 plants and the decreased plant size in *ddm1* F_1 and changing expression levels of these genes might be involved in the change of plant size.

Heterosis has been suggested to affect ET biosynthesis or signal transduction, and overexpression of the ET biosynthesis gene, *1-aminocyclopropane-1- carboxylate synthase 6* (*ACS6*), eliminated heterosis [34]. In this study, we made transgenic plants overexpressing eight genes encoding ET response factors but overexpressing these genes did not lead to any change of plant size, except for plants overexpressing *ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR22* (*ERF22*), which showed an increased plant size at 21 DAS. *Expansin* (*EXP*) and *Xyloglucan endotransglucosylases/hydrolase* (*XTH*) are known to be involved in loosening cell wall architecture and cell enlargement [37]. Upregulation of *XTH* genes has been observed in heterotic hybrids and hybrid mimic lines [17, 38]. In this study, overexpression of *β-expansin 1* (*EXPB1*) induced increased plant size at 14 DAS, while overexpression of *EXPB3* did not change the plant size. Overexpressing *XTH19* showed variation of plant size, and the average plant size was decreased. *XTH19* showed tissue specific expression, specifically in roots [39]. Constitutive *XTH19* expression in vegetative tissues may be negative for vegetative development. A growth-defense tradeoffs model has been proposed for heterosis or hybrid necrosis phenomena [1,40,41]. Overexpressing *CARBON/NITROGEN INSENSITIVE 1* (*CNI1)* or *Plant defensin 1.2b* (*PDF1.2b*) led to increased plant size at 21 DAS. *CNI1* is important for the carbon/nitrogen response during the early post-germinative growth, and overexpression of *CNI1* causes less sensitivity to change in C/N conditions [42]. *CNI1* expression was induced by *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*. DC3000) infection and overexpression of *CNI1* increased resistance to *Pst*. DC3000 [43]. In addition, overexpression of *CNI1* suppressed the senescence phenotype [44]. *PDF1.2b* encodes plant defensin and is involved in non-host pre-invasive defense response [45]. Most transcriptome analyses comparing heterotic inter- or intra-hybrids and their parents have shown the downregulation of defense responsive genes in F_1 plants [14,17,33,46,47]. However, upregulation of some defense response genes occurred in non-additively expressed genes, suggesting that upregulation of these genes might have a positive effect on plant growth. Overexpression of some genes, which were upregulated in big sized F_1 plants, showed an increased plant size at 14 or 21 DAS and a large variation of plant size within the lines. However, increased plant size was not observed at all time points. As plant size or heterosis is regulated by tissue- and stage-dependent transcriptional networks, a possible reason is that overexpression of a single gene is not enough to generate the phenotype and overexpression of multiple genes may be required. Alternatively, changes in gene expression at particular time points may be important for increased plant size.

4. Materials and Methods

4.1. Plant materials and growth condition

F_1 between C24 and Col accessions and its F_2 population were used for analysis of plant size, microarray, and qPCR. Plants were grown in a controlled environment (22 °C) under fluorescent lights (150–180 μmol photons·m−2·s−1) and a 16-h/8-h (day/night) photoperiod. Plants were grown in plastic dishes containing Murashige and Skoog (MS) agar medium supplemented with 1.0% sucrose (pH 5.7), and were transferred to soil at 14 DAS.

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4.2. Measuring seed size, rosette diameter, leaf area, and cell size

Dry mature seed was photographed under a stereoscopic microscope, and sizes were determined with Image-J software (http://rsb.info.nih.gov/ij/). Rosette diameter was measured for evaluation of plant size and equals the maximum diameter of the rosette as measured between the two largest leaves. Rosette diameter depends on leaf blade and petiole length. The 1st and 2nd leaves at 14 DAS were fixed in a formalin/acetic acid/alcohol solution (ethanol: acetic acid: formalin = 16: 1: 1). The leaf was photographed under a stereoscopic microscope, and sizes were determined with Image-J software. After examination of leaf area, they were cleared in a chloral hydrate/glycerol/water solution (chloral hydrate: H_2O: glycerol = 8: 2: 1), and the samples were photographed under Nomarski optics. The palisade cell number per fixed unit area in the subepidermal layer of the center of the leaf blade between the midvein and the leaf margin was counted. Three independent experiments were performed for examination of seed size, leaf area, and cell size. Statistical comparisons of seed size, leaf area, and cell size were performed using Student's *t*-test ($p < 0.05$).

4.3. Expression analysis

Total RNA was isolated from aerial tissues at 14 DAS in F_1 or F_2 plants using the SV Total RNA Isolation System (Promega). From 500 ng total RNA, first-strand cDNA was synthesized using random primers by SuperScript III Reverse Transcriptase (Invitrogen). Prior to qPCR, the specificity of the primer set for each gene was first tested by electrophoresis of PCR amplified products using EmeraldAmp MAX PCR Master Mix (Takara bio) on 2.0% agarose gel in which single products were observed. Absence of genomic DNA contamination was confirmed by the PCR of no RT control. RT-PCR conditions were 95 °C for 3 min followed by 30 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. qPCR was performed using a Rotor-Gene 3000 Real-Time Cycler (Qiagen). The cDNA was amplified using Platinum Taq DNA polymerase (Invitrogen). PCR conditions were 95 °C for 2 min followed by 40 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. Expression levels of genes were calculated relative to *Isopentenyl pyrophosphate-dimethylallyl pyrophosphate isomerase 2* (*IPP2*) genes using the comparative quantification analysis method with Rotor-Gene 6 (Qiagen). Data presented are the average and standard error (s.e.) from two or three biological and three experimental replications. Primer sequences are shown in Table S9.

4.4. Microarray analysis

Arabidopsis ATH1 Genome Array (Affymetrix) was used for transcriptome analysis. Total RNA (100 ng) from aerial tissues at 14 DAS from big and medium sized F_1 plants was used for probe synthesis. Biotinylated cRNAs were synthesized using the IVT Labeling Kit (Affymetrix). Hybridization and scanning were performed according to the manufacturer's instructions. Two independent biological replicates were performed. Data were analyzed following [14].

4.5. Gene ontology analysis

Analysis for enrichment of gene functional ontology terms was completed using the gene ontology (GO) tool agriGO [48]. The background reference for microarray analysis was the list of genes that displayed expression above-background in either the parental or F_1 samples from each platform [14]. Statistical tests for enrichment of functional terms used the hypergeometric test and false discovery rate (FDR) correction for multiple testing to a level of 5% FDR.

4.6. Constructs and plant transformation

The complete CDS were amplified by RT-PCR using gene-specific primers designed to add *Xba* I and *Sac* I or *Bam* HI and *Sac* I restriction sites to the 5′- and 3′-ends, and PCR products were cloned into pGEM T-easy vector (Promega). The DNA fragment was then inserted into *Xba* I and *Sac* I or

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Bam HI and *Sac* I restriction sites of the plant expression vector pBI121 under the control of CaMV35S promoter. The constructs were transformed into *Agrobacterium tumefaciens* strain EHA105 and transformation of Col accession was carried out by the floral dip procedure [49]. Positive transformants were selected in kanamycin (30μg/ml) plates and confirmed by PCR. Primers used for constructing the vector are listed in Table S9.

T_2 plants were grown in plastic dishes containing MS agar medium supplemented with 1.0% sucrose (pH 5.7). At 14 DAS, they were transferred to soil and grown under long day conditions (16 h light) at 22 °C. The presence or absence of transgene was examined by PCR using *neomycin phosphotransferase II* (*NPTII*) primer set (Table S9).

5. Conclusions

We found a variation in size in F_1 plants of *A. thaliana* and identified genes that were differentially expressed between big and medium sized F_1 plants. These differentially expressed genes tended to overlap with non-additively expressed genes in heterotic F_1, however, increases and decreases in expression levels in big sized plants/heterotic F_1 did not always match. Non-additively expressed genes showed tissue and stage specificity [1], e.g., upregulation of chloroplast-targeted genes was limited to a few days [14,18,19]. We suggest that changes in the expression of these genes, not the constitutively increased or decreased expression levels, may be important for increased plant size. Having variability in plant size in the F_1 generation is not a suitable phenotype for F_1 hybrid cultivars of crops, but this phenomenon may allow exploration of the factors necessary for maximizing the potential plant size or for stability of heterosis regardless of environmental effects.

Supplementary Materials: The following are available online, Figure S1: Expression pattern of genes upregulated in big sized F_1 plants compared with medium sized F_1 plants and downregulated in *ddm1* F_1 compared with wild type F_1, Figure S2: Plant phenotypes of transgenic plant lines of #9 and #19 at 21 DAS, Figure S3: Confirmation of transgene expression levels by RT-PCR, Table S1: Differentially expressed genes between big and medium sized F_1 plants, Table S2: Genes showing differential expression between big and medium sized F_1 plants and upregulation in C24/Col and C24/L*er* hybrids, Table S3: Validation of microarray expression data by quantitative RT-PCR, Table S4: Classification of gene expression pattern in big and medium sized F_1 plants, Table S5: Comparison of gene expression levels between big and medium sized F_2 plants, Table S6: GO function term overrepresented in genes showing B1&B2 > M1&M2 expression, Table S7: GO function term overrepresented in genes showing B1&B2 < M1&M2 expression, Table S8: GO function term overrepresented in genes showing B1&B2 > M1&M2 expression and upregulation in both C24/Col and C24/L*er* hybrids, Table S9: Sequences of primers used in this study.

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